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Inhibition of proliferative diseases by agents which are directed against PLK1 and
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Inhibition of proliferative diseases by agents which are directed against PLK1 and are selected from antisense oligonucleotides and duplex RNAs

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Inhibition of proliferative diseases by agents which are directed against PLK1 and are selected from antisense-oligonucleotides and duplex RNAs.

5

Specification

In eukaryotes, double-stranded (ds) RNA induces sequence-specific inhibition of gene expression referred to as RNA interference (RNAi). Since PLK1 (polo-like kinase 1) expression is elevated in a broad range of human tumors, RNAi was exploited to define the role of PLK1 for neoplastic proliferation.

After transfection Northern and Western blot analyses were used to examine the potential of 21-nt-long, short interfering (si) duplex RNAs targeted against human PLK1 for specific inhibition of PLK1 gene expression. Furthermore, the influence of dsRNAs on the phenotype and the proliferation of cancer cells was monitored.

Treatment of cancer cell lines (MCF-7 breast, HeLa S3 cervix, SW-480 colon and A549 lung) with appropriate dsRNAs resulted in a sequence-specific decrease in the level of mRNA and protein expressed from the human PLK1-gene. The analysis of mitotically arrested SW480 cells by fluorescence microscopy revealed centrosomes that lost their ability for microtubule nucleation. Moreover, dsRNA treatment against PLK1 resulted in a potent antiproliferative effect and apoptosis in tumor cells of different origin consistent with a powerful RNA silencing effect for the applied dsRNAs. In contrast, primary mammary epithelial cells exhibited low sensitivity towards PLK1-specific dsRNA. All statistical tests were two-sided.

30

These data indicate that PLK1 function is essential for centrosome-mediated microtubule events and consequently for spindle

assembly. Our observations implicate that dsRNAs targeted against human PLK1 may be valuable tools as antiproliferative agents that display activity against a broad spectrum of neoplastic cells at very low doses.

5 RNA-interference (RNAi) as part of a primitive immune system represents the ability of some viruses, transgenes or RNAs to trigger post-transcriptional degradation of homologous cellular RNAs (1,2). This mechanism has evolved to protect the genome of an organism against the hostile environment with dangerous opportunities for unwanted gene
10 expression and with parasites (transposons and viruses). Double-stranded RNA (dsRNA) has been shown to trigger sequence-specific gene silencing in numerous organisms such as nematodes, plants, trypanosomes, fruit flies and planaria. Studies in *C. elegans* and *Drosophila* revealed that a few molecules per cell are sufficient to eliminate a much larger pool of
15 endogenous mRNA and thereby induce a strong RNAi response suggesting a catalytic or amplification mechanism to contribute to gene silencing (3,4). After selection of a specific mRNA as target for RNAi, endogenous cleavage is a key step in degradation of the target mRNA. In *Drosophila* RNAi is independent of mRNA translation but requires ATP (5). During this
20 process dsRNA is reduced in size to fragments of 21-23 nucleotides by a ribonuclease III protein that is independent of the targeted mRNA. Subsequent cleavage of the mRNA was observed only within the region of identity with the dsRNA. In a recent study RNA duplexes of 21 nucleotides in length were shown to suppress gene expression in mammalian cell lines
25 (6). This report suggested that longer dsRNAs (50- and 500-bp) induced nonspecific reduction in reporter-gene expression probably as part of an interferon response (7).

30 Increasing knowledge about the genetic control of cellular proliferation provides the basis for the rational design of specific therapeutic strategies aimed at the regulation of proliferative disorders such as cancer. A key regulator for the mitotic progression in mammalian cells is the polo-like

kinase (PLK1) which is structurally related to the polo gene product of *Drosophila melanogaster*, Cdc5p of *Saccharomyces cerevisiae* and plo1⁺ of *Schizosaccharomyces pombe* (8). The plks from yeast, insects, amphibians and mammals represent a group of serine/threonine kinases that share a high degree of homology suggesting that the proteins have a close evolutionary and thereby functional relationship. PLK1 activity is elevated in tissues and cells exhibiting a high mitotic index including tumors (9,10). An increasing body of evidence suggests that the frequency of PLK1 expression is of prognostic value for patients suffering from different types of tumors like non-small cell lung cancer, squamous cell carcinomas of head and neck, melanomas, oropharyngeal carcinomas, ovarian and endometrial carcinomas (11). The importance of PLK1 as measure for the aggressiveness of a tumor results from its leading role for mitotic progression in particular the G₂/M transition (control of the mitosis promoting factor) (12). Moreover, PLK1 controls also additional steps during mitotic progression: regulation of the anaphase promoting complex and cytokinesis (13-15). Considering the potency of dsRNA for sequence-specific mRNA degradation in insect cells and the key role of PLK1 for mitosis, the current study focused on the inhibition of PLK1 expression in human cancer cells by RNA interference.

It was, therefore, an object of the present invention to provide a possibility to inhibit PLK1 activity.

In a first aspect of the present invention, this problem was solved by providing dsRNAs which are directed against PLK1 and their use for inhibiting PLK1 activity in the treatment of proliferative diseases and especially cancer diseases.

dsRNAs and antibodies

dsRNAs were synthesized and purified by Dharmacon Research Inc.

(Colorado, USA). The dsRNA sequences targeting PLK1 (NCBI accession number: X75932) correspond to positions 178-200 (dsRNA2), 362-384 (dsRNA3), 1416-1438 (dsRNA4) and 1570-1572 (dsRNA5) located within the open reading frame. dsRNA1 directed against lamin A/C (NCBI
5 accession number: X03444) represents positions 608-630 relative to the start codon. Such dsRNAs as well as their use for the treatment of proliferative disorders are considered especially preferred embodiments of the present invention.

10 The control dsRNA (scrambled) corresponds to the base composition of dsRNA4 as random sequence. Monoclonal PLK1 antibodies were obtained from Transduction Laboratories (Heidelberg) for Western blots and from Zytomed (San Francisco, USA) for kinase assays. Antibodies for actin were purchased from Sigma (Deisenhofen).

15 RNA preparation and Northern Blots

For isolation of total RNA an RNeasy-kit was used according to the manufacturer's protocol (Qiagen, Hilden). Radiolabeling of probes for PLK1 and β -actin was performed using 250 μ Ci of [α -P³²]dCTP (6000 Ci/mmol;
20 1Ci = 37 GBq) for each reaction, 50 μ M of each other dNTP and 10 pmol (each) of primer PLK1-17-low (5'-tgatgttggcacccttcagc-3') or actin-2-low (5'-catgaggtagtcagtcaggctc-3') as described previously (16). Northern blotting and hybridizations were carried out as reported (17).

25 Cell Culture

Ham's F12 and FCS were purchased from PAA, DMEM, RPMI 1640, PBS, OPTIMEM, oligofectamine, glutamine, penicillin/streptomycin and trypsin
30 from Invitrogen (Karlsruhe). Tumor cell lines SW-480, MCF-7 and HeLa S3 were provided by DSMZ (Braunschweig) and A549 by CLS (Heidelberg). Mammary epithelial basal medium (MEBM), growth medium supplements

(MEGM Single quotes) and the normal mammary epithelial cell system (HMEC) established from normal breast tissue were obtained from Clonetics (Verviers, Belgium). HMECs were passaged to select desired cells and cryopreserved in sixth passage, assured for fifteen population
5 doublings by the manufacturer. Cell culture was performed according to the supplier's instructions.

Western Blot Analysis

10 48 hrs after dsRNA treatment cells were lysed for subsequent Western blotting (16). Membranes were kept for 1 hr with monoclonal antibodies for PLK1 (1:250) and actin (1:200.000) followed by incubation with goat anti-mouse serum (1:2.000) for 30 min. Western blots were performed as described (18).

15 Kinase Assays

48 hrs after dsRNA treatment cells were lysed, endogenous PLK1 was immunoprecipitated using monoclonal PLK1 antibodies, incubated with
20 0.5-1 μ g of substrate and 2 μ Ci of [γ -P³²]ATP for 30 min at 37°C in kinase buffer (20 mM HEPES pH 7.4, 150 mM KCl, 10 mM MgCl₂, 1 mM EGTA, 0.5 mM DTT, 5 mM NaF, 0.1 mM Na₃VO₄) and products of kinase reaction were fractionated on a 12% SDS-polyacrylamid gel (BioRad, München).

25 Indirect Immunofluorescence

Cells were stained as described before (18). Antibodies were used as follows: polyclonal rat α -tubulin (Serotec/Biozol, Eching) 1:100, polyclonal rabbit PLK1 (19) 1:100 or monoclonal γ -tubulin (Sigma) 1:100. Cells were
30 analyzed with a confocal laser scan microscope (CLSM) using a 100x oil immersion objective.

FACS analysis

Analysis of cell cycle and apoptosis was carried out using a Becton Dickinson FACScan. Cells were harvested, washed with PBS and probed
5 with CycleTEST™ PLUS DNA reagent kit (Becton Dickinson) according to the manufacturer's protocol. For apoptosis detection cells were harvested 48 hrs after transfection, fixed with ice-cold 70% ethanol, treated with RNase A (5 µg/ml) and stained with propidium iodide (50 µg/ml). Subsequent analyses were performed using the CELLQuest software.

10

In vitro administration of dsRNAs and determination of cell proliferation

After transfection of cancer cells (oligofectamine method) the inhibitory activity of dsRNA1-5 on cell proliferation was tested. In brief, 1 day before
15 transfection cells were split to a density of 5×10^5 /25cm²-culture flask corresponding to a density of 40-50% at the time of transfection. Cells were treated with dsRNA2-5 (PLK1) or dsRNA1 (lamin) as internal negative control at a concentration of 56 nM or in the dose-dependence experiments with concentrations of 0.5 nM-2 µM. Incubation with dsRNAs
20 in OPTIMEM with oligofectamine at 37°C for 4 hrs was followed by addition of fresh medium with threefold FCS. At the appropriate time after dsRNA treatment cell numbers were determined using a hemacytometer. Cell viability was assessed by trypan blue staining. Each experiment was performed in triplicate and the standard deviation for each group was
25 determined.

Quantitative analysis

Quantification of PLK1 and actin levels was performed using a Kodak gel
30 documentation system (ID 3.5). To determine Northern and Western blot signal intensities, autoradiographs were scanned. Integration of signal intensities was followed by quantitative comparison of PLK1 and actin

expression. The expression values were given in percentage of control.

Statistical methods

- 5 Statistical analysis was performed with two-sided paired t-test and p-values were calculated compared to untreated control.

dsRNAs inhibit the expression of PLK1 mRNA and protein specifically

- 10 Because RNAi acts by decreasing the half-life of RNA, the natural stability of RNA will have a quantitative influence upon its suitability as target for gene silencing. Thus, we started our study by testing dsRNAs for their ability to reduce the endogenous level of PLK1 mRNA in different cancer cell lines (MCF-7 breast, HeLa S3 cervix, SW-480 colon and A549 lung).
- 15 Four duplex RNAs, each 21-nt in length (dsRNA2-5) with symmetric 3' overhangs of 2 deoxythymidines, directed against the open reading frame of PLK1 were tested for their potential of PLK1-specific interference in tumor cells. As an internal negative control a duplex RNA targeted against lamin (dsRNA1) was used. Northern blot analyses standardized to the
- 20 expression of actin were performed. Treatment of MCF-7 breast cancer cells in vitro with dsRNA2-5 at a concentration of 56 nM in the presence of oligofectamine did not reduce PLK1 mRNA 6 hrs after transfection significantly but led to an essential loss of PLK1 mRNA within 24 hrs and 48 hrs (Fig. 1 A). The study revealed that all tested dsRNAs targeted
- 25 against PLK1 led to reduced PLK1 mRNA levels in human MCF-7 cells to levels of 20-30% compared to untreated cells. Suppression by dsRNA2-5 could not only be demonstrated for MCF-7 cells but also for other tumor cell types like HeLa S3 and SW-480 (Fig. 1 B, C). In SW-480 cells the reduction of PLK1 mRNA occurred rather rapidly within 6 hrs but was only
- 30 apparent for dsRNA4 and dsRNA5. Suppression by dsRNA4 was also observed in A549 cells (Fig. 1 D).

Subsequently, we determined whether decreases in PLK1 mRNA were accompanied by corresponding reduction of PLK1 protein. The expression of PLK1 protein in different tumor cell lines was evaluated 48 hrs after single application of dsRNAs. Significant inhibition ($p < 0.001$) of the 68 kDa-PLK1 protein in MCF-7 cells was observed with dsRNA4 and dsRNA5 (87 and 79%, respectively), which were previously shown to reduce PLK1 transcript levels significantly (Figs. 1A, 2A). A reduction of PLK1 mRNA induced by the application of dsRNA3-5 correlated also to a significantly lower level of the corresponding protein in HeLa S3 (dsRNA3: 81%; $p < 0.001$; dsRNA4: 65% and dsRNA5: 73%; $p < 0.01$), SW-480 (dsRNA4: 82% and dsRNA5: 92%; $p < 0.001$) and A549 cells (dsRNA3: 70%; $p < 0.01$; dsRNA4: 84% and dsRNA5: 65%; $p < 0.001$) (Fig. 2B-D).

To test for the specificity of the dsRNA action, dsRNA1 (control) directed against lamin was evaluated for its potential to alter expression of PLK1 or actin. The levels (mRNA, protein) were not influenced significantly proposing that the above described effects on cells treated with dsRNAs are sequence-specific for PLK1. This notion gained further support from the observation that dsRNA2-5 did not alter the expression of actin mRNA either. Furthermore, the concentration of dsRNA4 correlated to protein levels of PLK1 (Fig. 2E). The treatment of MCF-7 cells with dsRNA4 at concentrations between 5.6 and 56 nM exhibited a pronounced inhibitory effect on PLK1 protein levels (inhibition of 81%). Increasing the concentration to 566 nM diminished the inhibitory potential of dsRNA4. On the other hand reducing the concentration to 0.5 nM did not increase the level of PLK1 protein.

Immunoprecipitated PLK1 from MCF-7 cells treated with dsRNA for 48 hrs was subjected to kinase assays to directly assess whether downregulation of PLK1 protein level correlates also to reduced PLK1 kinase activity. In dsRNA4-treated cells phosphorylation of the cytoplasmic retention signal within human cyclin B1 as exogenous substrate (12) was reduced to 18%

of the level in control cells (data not shown). Taken together, these results suggest that PLK1 mRNA and protein were specifically reduced in dsRNA-treated cancer cells correlating with low levels of PLK1 activity.

5 Reduced levels of PLK1 protein result in abrogation of spindle formation

In previous studies microinjection of PLK1-specific antibodies induced abnormal distribution of condensed chromatin and monoastal microtubule arrays that were nucleated from duplicated but unseparated chromosomes
10 (20). Here we focused on analyzing the phenotype of dsRNA-treated cells that exhibit a major downregulation of PLK1 expression in cancer cells. Antibodies directed against α -tubulin to visualize the spindle apparatus and γ -tubulin to localize centrosomes were used for staining 48 hrs after transfection. While untreated control cells proceeded through mitosis, cells
15 incubated with dsRNA4 arrested in different mitotic stages depending on the cell type. SW-480 cells treated with dsRNA4 did not enter prophase as can be derived from the lack of prophase typical chromosome condensation in the nuclei of DAPI-stained cells. However, many cells were found with separated centrosomes which moved to opposite ends of the
20 nucleus (Fig. 3A upper panel; γ -tubulin). Centrosomes were devoid of any microtubule connection (Fig. 3A upper panel; α -tubulin). According to their DAPI-fluorescence nuclei contained 4N DNA although no chromatin condensation was detected. Thus, while the centrosomes performed prophase-typical separation, nuclei seem to persist in G2-phase. For
25 comparison, in untreated controls centrioles in this early state of prophase organized astral microtubules (Fig. 3A lower panel) and displayed chromosome condensation in the nucleus.

MCF-7 cells on the other hand displayed numerous apoptotic nuclei in
30 DAPI-stained cultures but no mitotic stages after dsRNA4 treatment. Examination of the supernatant of these cultures revealed a high percentage of mitotic cells which obviously lost substrate adhesion.

Metaphase or telophase chromosomal arrangements were rarely identified. More than 90% of all mitotic stages were characterized by highly condensed, knob-like chromosomes which remained in an overall structure resembling the shape of a nucleus (Fig. 3B). Only a few chromosomes left
5 this ensemble lying in the immediate vicinity. This phenotype indicates that the nuclear envelope has disappeared but no further, mitotic spindle-related arrangement of chromosomes occurred. Chromatid separation did not take place. γ -tubulin was distributed all over the cytoplasm of these cells which also did not contain any microtubules. The lack of microtubules could be a
10 consequence of cell death after loss of adhesion. In contrast, primary epithelial cells (HMEC treated with dsRNA4 at the same concentration proceeded through mitosis with normal phenotype.

dsRNA treatment induces G₂/M cell cycle arrest and apoptosis in cancer
15 cells in vitro

FACScan-analysis was performed to determine the role of cell cycle arrest for the death of dsRNA-treated cells. A strong G₂/M arrest was detected: SW-480 (fivefold), MCF-7 (threefold), HeLa S3 (fivefold) and A549 cells
20 (twofold) (Fig. 4A). CLSM experiments revealed an elevated number of apoptotic cells with disintegrated nuclear membranes and condensed chromatin (Fig. 4B). To address this observation in more detail, we asked the question whether gene silencing of PLK1 causes apoptosis in different tumor types. The percentage increase of Sub2N DNA content was
25 determined with FACScan analysis. While control cells exhibited between 1 and 5% Sub2N DNA, PLK1-specific dsRNA4 induced increased Sub2N DNA in SW-480 (17%), MCF-7 (33%), HeLa S3 (50%) and A549 cells (13%) 48 hrs after transfection. This phenomenon can be explained with
30 activation of interferones because dsRNAs are known to induce IFN α/β and to be pro-apoptotic agents (7).

Furthermore, the sensitivity of primary human mammary epithelial cells

(HMEC) towards dsRNAs was evaluated. In contrast to the cancer cells tested, primary cells did not exhibit G₂/M arrest after treatment with 56 nM dsRNA4. dsRNAs did not induce an apoptotic phenotype and did not increase the ratio of Sub2N DNA significantly suggesting a differential effect of dsRNA in cancer cells compared to normal cells.

PLK1-specific dsRNAs inhibit the growth of cancer cells in vitro

MCF-7 cells were treated with dsRNA1-5 at a concentration of 56 nM to determine whether dsRNAs influence the proliferation of tumor cells. The growth rate of 5×10^5 cells was determined over a period of four days. While the treatment with the transfection agent (oligofectamine) alone had no effect on the growth rate of MCF-7 cells, treatment with a control dsRNA1 (lamin) had only limited effect (Fig. 5A). In contrast, dsRNA2-5 had a significant antiproliferative effect compared to untreated MCF-7 cells within 96 hrs (dsRNA2: 83%; $p < 0.01$; dsRNA3: 81%; dsRNA4: 97%; dsRNA5: 89%; $p < 0.001$ for dsRNA3-5) (Fig. 5A). Specificity of the dsRNA-mediated inhibition was also indicated by a dose-dependent reduction of cell growth (Fig. 5B). Treating MCF-7 cells with dsRNA1 (lamin) or with a scrambled control dsRNA demonstrated that both dsRNAs did not reduce cell numbers significantly in any concentration tested compared to oligofectamine. In contrast, increasing concentrations of dsRNA4 (5.6-566 nM) led after 48 hrs to almost complete cell death (Fig. 5B). It was subsequently examined whether the reduction of proliferative activity is cell type-specific or can also be achieved in other cancer cell lines. dsRNA4 and dsRNA5 were shown to inhibit cell growth also significantly in SW-480 ($p < 0.01$), HeLa S3 ($p < 0.001$) and in A549 cells ($p < 0.05$) suggesting that PLK1 expression is essential for the proliferation of different types of cancer cells (Fig. 5C-E).

Subsequently, the growth behavior of the tumor cell line MCF-7 was compared to normal mammary epithelial cells (HMEC). To gain the same

extent of growth reduction in primary epithelial cells (HMEC), a 350fold higher concentration of dsRNA4 was required compared to MCF-7 cancer cells. While in MCF-7 cells a growth reduction of 38% could be achieved with a dsRNA4 concentration of 5.6 nM, in HMECs for a growth reduction of 31% 2 μ M were required (Fig. 5F). This observation suggested an elevated sensitivity of cancer cells towards dsRNA directed against PLK1 compared to primary epithelial cells.

Recent observations shed light on double-stranded RNA as a tool for the 'knock-down' of gene expression in a number of organisms (1,2). RNAi seems to be important for many developmental and antiviral mechanisms. Still, the analysis of RNAi in *C. elegans* revealed target RNAs that partially or fully resist post-transcriptional gene silencing (16). While reduction of reporter gene expression in *Drosophila* S2 cells was almost complete, specific suppression in mammalian cells was either less pronounced or could not be achieved (6). These observations raised the question whether RNA silencing is a general phenomenon in mammalian cells. Thus, the purpose of the present study was to explore the potential use of duplex RNAs for the inhibition of human cancer cells. In addition, we intended to evaluate the role of PLK1 for the proliferation of tumor cells. Our study demonstrated that administration of dsRNAs targeted against human PLK1 reduced the level of PLK1 transcripts in cell culture. dsRNA4 exhibited a pronounced inhibitory effect at a concentration of 5.6 nM. The effect disappeared if the dsRNA concentration was reduced below 0.5 nM. One of the most attractive features of dsRNA-based gene silencing is the potent inhibitory effect at low concentrations. In comparison, phosphorothioate antisense oligonucleotides display IC₅₀ values between 100 and 500 nM (21). Thus, the limitation of phosphorothioate antisense oligonucleotides as pharmacological agent due to the potential toxicity seems to be less likely in the case of dsRNA, because efficient 'knock-down' of target genes can be achieved with very low concentrations. Moreover, we paid considerable attention to the specificity of administered dsRNA. First, within a small set

of tested dsRNAs targeted to different regions of human PLK1 only certain candidates had a potent silencing effect. Since the mechanism of RNA silencing is still under vivid investigation, we assume that silencing efficiency could reflect several parameters such as tissue-specific effects, RNA stability and homeostatic regulation, which can answer a decrease in mRNA concentration with a metabolic compensation that would restore expression levels. Second, a scrambled dsRNA (permuted sequence of dsRNA4) or a dsRNA targeted to lamin had only little effect on the level of PLK1 mRNA. Non-specific reduction of mRNA expression was expected as part of the interferon response in living cells (7). Four tumor cell types were responsive to the antiproliferative effects of dsRNA4 (MCF-7 breast, HeLa S3 cervix, SW-480 colon and A549 lung), supporting the premise that PLK1 silencing might be useful for the treatment of tumors in future in vivo experiments. This view gained attractive support by the observation that primary epithelial cells were not suppressed by dsRNA at concentrations that extinguished tumor cells. Thus, toxic side-effects in normal cells exerted by dsRNA targeted to human PLK1 are less likely. Taken together, these observations strongly suggest that the inhibitory effect of dsRNA4 on PLK1 expression and the biological consequences that appear to result from these inhibitory effects in cell culture occur through an RNA silencing mechanism.

The ability to use duplex RNA to target selectively genetic mechanisms involved in tumorigenesis gives rise to the fascinating chance that these novel agents could be used, not only as a new class of chemotherapeutic agents to combat cancer, but also to gain a better understanding of the critical molecular events responsible for initiating and maintaining the cancer phenotype. The above results raise some intriguing questions relative to the role of PLK1 in cancer cells. Centrosomes play a critical role in generating genetic instability in cancer cells (22,23). They contribute to spindle abnormalities and disturbed chromosome segregation, which is often accompanied with profound alterations in key cellular functions like

apoptosis, cell cycle progression, control of cell cycle checkpoints and cell growth regulation. Different studies tested the impact of PLK1 on the function of mammalian centrosomes (20,23-25). While the analysis of HeLa cells microinjected with PLK1-specific antibodies revealed monoastral microtubule arrays that were nucleated from duplicated but unseparated centrosomes, RNA silencing allowed to separate centrosome division from microtubule anchoring: Centrosomes still divided and separated from each other but obviously without microtubule interaction. If the pericentriolar matrix surrounding centrioles becomes dissolved in early prophase, centrosomes are not kept in close proximity any longer. Lack of PLK1 after dsRNA treatment prevents the formation of the microtubule nucleation complex required for aster and spindle formation.

In MCF-7 cells centrioles could not organize microtubules because chromosome arrangements other than those typical for late interphase/early prophase nuclei are very rare. While SW-480 cells arrest predominantly in G₂/prophase, MCF-7 cells proceed further to pro-/prometaphase characterized by condensed chromosomes and beginning nuclear envelop breakdown. This phenotype is a consequence of missing spindle formation and retarded chromosome separation (26). Thus, 'knock-down' of PLK1-function may induce different mitotic phenotypes at various stages due to varying checkpoint control in different cancer cells or might alternatively correlate to the endogenous level of PLK1 mRNA in tumor cells reflecting a mutation-sensitive interaction of PLK1 mRNA and Hsp90 (27).

Taken together, although, the mammalian genome contains several polo-related kinases, the 'knock-down' of PLK1 alone by RNA silencing is sufficient to prevent centrosome-induced spindle formation and to induce apoptosis in different tumor cells. Extra centrosomes in cancer cells might lead to chromosome missorting and damage causing aneuploidy which may induce the loss of tumor suppressor genes or activate oncogenes. Recent

observations pointed out that centrosomal abnormalities can be detected in early forms of human breast and prostate cancer (28). This might suggest that centrosomes are the driving force of cancer formation instead of being a consequence of it. Our cell culture experiments indicate that gene
5 silencing of PLK1 inhibits centrosome function to an extent which induces apoptosis of cancer cells. Future experiments might also shed light on new techniques aimed at restoring normal centrosome function by regulating PLK1 activity. Furthermore, investigations examining the effects of
dsRNA4 against tumors in Xenograft experiments are of obvious
10 importance.

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FIGURE LEGENDS

Fig. 1. Reduction of PLK1 mRNA in cancer cells. (A) Northern blot analysis of PLK1 mRNA in MCF-7 cells 6, 24 and 48 hrs after dsRNA treatment. To ascertain uniformity of loading membranes were reprobed with human β -actin. Indicated are signals for PLK1 and actin in all autoradiographs. Percent inhibition was calculated by comparison with PLK1 mRNA levels of cells grown in the absence of dsRNAs. PLK1 mRNA expression in HeLa S3 (B), SW-480 (C) and A549 cells (D) 6, 24 and 48 hrs after dsRNA application.

Fig. 2. Inhibition of PLK1 protein expression by PLK1-specific dsRNAs. Percent inhibition was calculated by comparison with PLK1 protein levels in cells grown in the absence of dsRNAs. (A) Western blot analysis of PLK1 protein in MCF-7 cells 48 hrs after dsRNA treatment. Indicated are the signals for PLK1 and actin. Actin served as control for equal loading (upper panel). Mean values of at least 3 independent experiments with standard deviation are shown (lower panel). Quantification of PLK1 protein in HeLa S3 cells (B), SW480 cells (C) and A549 cells (D) after normalization to actin. (E) PLK1 protein correlated to the concentration of dsRNA4 in MCF-7 cells.

Fig. 3. dsRNA4 treatment resulted in abrogated spindle formation at centrosomes in SW-480 cells and disturbed chromosome phenotypes in early mitosis of MCF-7 cells. Cells were transfected with dsRNA4 and immunostained 48 hrs post transfection for γ - (green), α -tubulin (red) and DAPI (blue). (A) dsRNA4-treated SW-480 cells with missing spindles at centrosomes. (upper panel); arrows: cell with centrosomes devoid of any mikrotubule connection; untreated cells with normal spindle formation (lower panel). (B) Supernatant of MCF-7 cell cultures treated with dsRNA4 contained many mitotic cells. Most of them were characterized by highly

condensed, knob like chromosomes. Some chromosomes are located adjacent to the nucleus (arrows).

5 Fig. 4. dsRNA treatment induced G₂/M cell cycle arrest and apoptosis in cancer cells. (A) FACScan analysis demonstrated strong G₂/M arrest in SW-480, MCF-7, HeLa S3 and A549 cells. (B) Confocal laser scanning microscopy revealed apoptotic cells with disintegrated nuclear membranes and condensed chromatin (phase contrast image).

10 Fig. 5. Antiproliferative effects of PLK1-specific dsRNA-mediated inhibition in cancer cells. dsRNA against lamin served as internal negative control. Percent inhibition was calculated by comparison to the number of cells grown in the absence of dsRNAs. (A) Growth inhibition of MCF-7 cells over a period of 4 days. (B) Dose-dependent reduction of MCF-7 cells 24, 48
15 and 72 hrs after dsRNA4 treatment compared to dsRNAI- or scrambled dsRNA-treatment. Growth inhibition of SW-480 (C), HeLa S3 (D) and A549 cells (E) over a period of 4 days. (F) Growth of normal mammary epithelial cells (HMEC over a period of 4 days after treatment with either dsRNAI, dsRNA4 or scrambled dsRNA in different concentrations (56 nM-2 μ M).

20

A central role for polo-like kinases (PLK) in regulating several stages of mitotic progression has been born out in several species. Overexpression of PLK1 is observed in the majority of hitherto analyzed human tumors. PLK1 overexpression is a negative prognostic factor in patients suffering from
25 non-small cell lung cancer, head and neck tumors, esophageal carcinomas and melanomas. In order to define the role of PLK1 for mitotic progression of human cells and for neoplastic cell growth, phosphorothioate antisense oligonucleotides (ASOs) were tested to selectively downregulate PLK1 expression in MDA-MB-435 (breast cancer), HeLa S3 (cervical carcinoma)
30 and A549 (non-small cell lung cancer) cells. ASOs were identified which suppress PLK1 mRNA and protein in a dose-dependent and sequence-specific manner. This approach also led to reduced PLK1

serine/threonine kinase activity. Downregulation of cellular PLK1 levels in cancer cells altered cell cycle progression moderately with an elevated percentage (20-30%) of cells in G₂/M. Furthermore, cells with reduced PLK1 protein gained a rounded phenotype with multiple centrosomes. Moreover, ASO treatment resulted in potent antiproliferative effects in cell culture. Considerable antitumor activity was observed in vivo against A549 cells. This study suggests that antisense inhibitors targeted against PLK1 at well tolerated doses may be considered as a cancer therapeutic agent.

The mammalian polo-like kinase (PLK1) is structurally related to the polo gene product of *Drosophila melanogaster*, Cdc5p of *Saccharomyces cerevisiae* and plo1⁺ of *Schizosaccharomyces pombe*. This emerging family of serine/threonine kinases is highly conserved from yeasts to humans. The ability to regulate multiple stages of the mitotic progression is the hallmark of polo-like kinases (plks). Detailed information about the role of plks for mitotic progression came from genetic studies on *Drosophila* polo, the founding member of the family. *Drosophila* mutants homozygous for a strong mutant polo allele die as larvae (Llamazares et al., 1991; Sunkel et al., 1988). Flies with a weaker mutant allele produce embryos with severe mitotic defects such as condensed chromosomes with irregular microtubule arrays and a lack of organized centrosomes. Additional genetic approaches provided further insight into the function of the putative yeast homologues of polo. In *Saccharomyces cerevisiae* mutations in the CDC5 gene cause abnormalities in both mitotic and meiotic divisions (Byers et al., 1974; Hartwell et al., 1973; Sharon et al., 1990). CDC5 mutants arrest in late mitosis as large, budded cells with partially segregated nuclei on an elongated spindle (Kitada et al., 1993). Furthermore, loss of plo1⁺ function in *Schizosaccharomyces pombe* has two consequences: It leads to either a mitotic arrest in which condensed chromosomes are associated with a monopolar spindle or following the completion of nuclear division to a failure in septation (Ohkura et al., 1995). Taken together, deletion or strong mutations in plk-coding genes of different species cause severe

growth retardation or even cellular lethality suggesting that plks play key roles for the mitotic progression of lower eukaryotes.

Whereas the yeasts can achieve cell-cycle progression utilizing a single
5 cyclin-dependent kinase, mammals have evolved multiple forms of both
Cdk's and their activating Cdc25 phosphatases. The phylogenetic
development of the plk family is comparable. In contrast to lower
eukaryotes, which possess only one plk gene, at least three PLKs (PLK1,
10 PLK21,5NK, PLK31FNK) could be isolated from mammalian cells (Clay et
al., 1993; Golsteyn et al., 1994; Holtrich et al., 1994; Holtrich et al.,
2000; Lake et al., 1993; Li et al., 1996; Simmons et al., 1992). Their
specific functions are very complex and in particular their ability to
complement each other remains to be elucidated. We identified human
15 PLK1 and could demonstrate that levels of PLK1 transcripts and protein are
elevated in tissues and cells exhibiting a high mitotic index including
tumors and immortalized cell lines (Clay et al., 1993; Golsteyn et al., 1994;
Holtrich et al., 1994; Lake et al., 1993; Yuan et al., 1997). An increasing
body of evidence suggests that the frequency of PLK1 expression is of
20 prognostic value for patients suffering from different types of tumors like
non-small cell lung cancer, squamous cell carcinomas of head and neck,
melanomas and esophageal carcinomas (Knecht et al., 1999; Strebhardt et
al., 2000; Tokumitsu et al., 1999; Wolf et al., 1997). The importance of
PLK1 as measure for the aggressiveness of a tumor and its key role for
cellular proliferation raise the question whether the inhibition of the PLK1
25 function in mammalian cells induces as dramatic effects as previously
observed in lower eukaryotic cells.

Increasing knowledge about the genetic control of cellular proliferation
provides the basis for the rational design of specific therapeutic strategies
30 aimed at the regulation of proliferative disorders such as cancer. Although
certain unanswered questions concerning the applicability of the antisense
technology remain (Stein, 1995; Wagner, 1995), this experimental

approach can, when targeted to key elements of proliferation-relevant signaltransduction pathways, prevent the development of specific human cancers. Several phosphorothioate antisense oligonucleotides (ASOs) are currently being evaluated in patients suffering from different types of cancer such as ovarian, colon, lymphoma and melanomas (Crooke, 2000).

Furthermore, studies in which these drugs are used in combination with traditional chemotherapeutic agents are in progress.

To elucidate the role of PLK1 for the inhibition of tumor cell growth, in the present study we tested the potential of phosphorothioate ASOs targeted against human PLK1 to inhibit its mRNA and protein expression. We then evaluated the effects of PLK1-specific ASOs on the proliferative activity of human tumor cells (breast cancer, MDA-MB-435; non-small cell lung cancer, A549 and cervical carcinoma, HeLa S3) in vitro and in vivo to shed light on the role of PLK1 as target for cancer treatment.

The work leading to the present invention therefore also provides for phosphorothioate ASOs targeted againsts PLK1 and their use for treatment of proliferative diseases like for example cancer diseases. Such phosphorothioate AOS are further subject matters of the present invention as well as their uses.

ASOs inhibit specifically the expression of PLK1 mRNA and protein

26 phosphorothioate ASOs, each 20 nucleotides in length and predicted to hybridize with human PLK1 mRNA were tested to identify effective candidates capable of inhibiting PLK1 gene expression in human tumor cells. These ASOs were homologues to different regions of PLK1 mRNA, with 9 ASOs targeted to the 5'-untranslated region, 6 targeted to sites within the coding region of PLK1 and 11 targeted to the 3'-untranslated region (Fig. 6 a). Binding of ASOs to the complementary sequence of the

mRNA for a specific gene results in gradual downregulation of the protein and loss of function of that gene mostly due to the activation of RNase H, which cleaves the mRNA at RNA/DNA duplex sites (Dirksen et al., 1981). Since phosphorothioates are excellent substrates for RNase H, antisense
5 activity for each of these ASOs was evaluated with Northern blot analyses using a PLK1-specific probe.

Treatment of MDA-MB-435 breast cancer cells in vitro with ASOs against PLK1 at a concentration of 250 nM in the presence of uptake-enhancing
10 cationic lipids (DOTAP) led in few cases to an essential loss of PLK1 mRNA within 24 hrs as demonstrated in figure 6b. The evaluation of Northern blots standardized to the expression of actin or glyceraldehyde-3phosphate dehydrogenase (G3PDH) mRNA revealed that the ASOs, named P12 and P13, which target the 3'-untranslated domain, are efficient inhibitors for
15 reducing PLK1 mRNA in cultured human MDA-MB-435 cells to levels of 30% and 40%, respectively compared to DOTAP-treated cells. Still, most tested ASOs had limited or no influence on the level of endogenous PLK1 mRNA. Furthermore, two control ASOs (HSV-ASO derived from Herpes Simplex Virus and a nonsense-ASO representing a random sequence) led
20 only to a weak reduction. Specific reduction by the ASOs P 12 and P 13 could not only be demonstrated for MDA-MB-435 cells but also for other tumor cell types like HeLa S3 and A549 (Fig. 6 c, d).

To determine whether decreases in PLK1 mRNA levels induced by ASOs
25 were accompanied by corresponding reduction in protein levels, Western blot analyses were performed to evaluate the expression in different tumor cell lines after single application of ASOs. Significant inhibition ($p < 0.001$) of the 68 kDa-PLK1 protein in MDA-MB-435 cells was observed with the ASOs P12 (80%) and P13 (73%) which were previously shown to be
30 potent inhibitors of gene expression (Fig. 7a). A reduction of PLK1 mRNA induced by the application of P12 and P13 resulted also in a significantly lower level ($p < 0.05$) of the corresponding protein in HeLa S3 (P12: 87%;

P13: 47%) and A549 cells (P12: 74%; P13: 61 %) (Fig. 7b, c).

Specificity of the ASO action requires that the outcome is dose-dependent and proportional to the downregulation of the gene. To test for specificity, the concentration of P12 and a control ASO (HSV) was correlated to protein levels of PLK1. The treatment of HeLa S3 and MDAMB-435 cells with P12 exhibited a dose-dependent reduction of PLK1 expression with a median inhibitory concentration (IC₅₀) of 50-75 nM (Fig. 2d, e). The control ASO (HSV) had no significant effect in Western blot analyses. The expression of other cellular proteins such as actin and p38 (MAP kinase family) was not influenced significantly indicating that the effects on cells treated with P 12 are sequence-specific for PLK1.

To directly assess whether downregulation of PLK1 protein level correlates also to reduced PLK1 kinase activity, immunoprecipitated PLK1 from HeLa S3 cells was subjected to enzymatic tests. Figure 2f shows the kinase activity of PLK1 precipitated with immune sera from P12-, control ASO (HSV)- and untreated cells. Although approximately equal amounts of PLK1 protein were present in the three immunoprecipitates, in P12-treated cells phosphorylation of exogenous casein was reduced to 50% of the enzymatic level in control cells. Virtually no kinase activity was detected in the preimmune precipitate (data not shown). These results suggest that the amount of PLK1 protein was specifically reduced in P12-treated cancer cells correlating with low levels of PLK1 activity.

PLK1-specific ASOs inhibit the growth of cancer cells in vitro

It was of particular interest to determine whether ASOs which reduce PLK1 expression influence the proliferation rate of tumor cells. For this purpose MDA-MB-435 cells were treated once with either P12, P13 or a control ASO (HSV) at a concentration of 250 nM. The growth rate of 5×10^5 cells was determined over a period of 2 days. The treatment with the lipofection

agent (DOTAP) alone or a control ASO (HSV) had only limited effect on the growth rate of MDA-MB-435 cells. In contrast, P12 and P13 had a significant antiproliferative effect ($p < 0.05$) of approximately 98% and 93% compared to DOTAP-treated MDA-MB-435 cells within 48 hrs (Fig. 8a). In addition, the remaining 24 ASOs, which had been tested for their ability to reduce the endogenous level of PLK1 mRNA, were utilized to measure their potential to influence the growth of MDA-MB-435. The analysis revealed that those ASOs, which induced only a slight reduction of cellular mRNA, had only limited inhibitory effect on the proliferation of the breast cancer cell line MDA-MB-435 (data not shown). Whether the reduction of proliferative activity is cell type-specific or can also be achieved in other cancer cell lines was subsequently examined. P12 and P13 were shown to determine the extent of growth inhibition also significantly in HeLa S3 cells ($p < 0.001$) and in A549 cells ($p < 0.05$) suggesting that PLK1 expression is essential for the proliferation of different types of cancer cells (Fig. 8 b, c). Additional evidence for the specific activity of the inhibitory ASO P12 came from a dose-dependent reduction of cell growth in MDA-MB-435 and in HeLa S3 cells (Fig. 8d, e). Increasing concentrations of P12 (50-250 nM) reduced the number of MDA-MB-435 cells by 80% and the number of HeLa S3 cells by 95% within 24 hrs compared to control cells. While maximal protein reduction was achieved in HeLa S3 cells with 100 nM (Fig. 7d), a concentration of 250 nM was required for MDA-MB-435 cells. In both cell lines 250 nM were necessary for maximal growth inhibition.

Reduced levels of PLK1 protein induce mitotic abnormalities

To determine whether the death of ASO treated cells was associated with cell cycle arrest or phenotypic abnormalities, we used FACS-analysis and laser scanning microscopy. Flow cytometric analyses of all three cell lines demonstrated that the induction of the G₂/M cell cycle arrest was moderate. The ASO treatment resulted in an increase of cells in G2/M Of

approximately 20-30% (data not shown).

Previous observations have demonstrated that microinjection of
PLK1-specific antibodies resulted in abnormal distribution of condensed
chromatin and monoastal microtubule arrays that were nucleated from
duplicated but unseparated chromosomes (Lane et al., 1996). Here we
showed for the first time the reduction of endogenous PLK1 protein and
corresponding PLK1-kinase activity in cancer cells. To analyze cell cycle
phenotypes of P12-treated cells under these conditions, we immunolabeled
A549 cells. Antibodies directed against α -tubulin to visualize the spindle
apparatus and γ -tubulin to localize centrosomes were used 48 hrs after
transfection with P12 and HSV-ASO, respectively. While most of the
HSV-ASO-treated cells proceeded through mitosis without abnormal
phenotypes, many cells incubated with P12 gained a rounded shape and
lost adherence to the cell culture plastic. Whereas application of
HSV-ASOs preserved normal centrosome maturation in A549 lung cancer
cells, P12-treated cells displayed often unseparated chromosomes and
multiple centrosomes (Fig. 9). Partially separated chromosomes induced
the formation of nuclear membranes enclosing at least one centrosome.
This led to abnormal distribution of centrosomes in interphase cells as
demonstrated in figure 9 (a, b).

In vivo antitumor activity of PLK1 ASOs

Having demonstrated that PLK1 ASOs are capable of inhibiting growth of
cancer cells in culture in a target- and sequence-specific manner, we tested
whether P12 and P13 have also an inhibitory capability in vivo. For this
purpose tumor fragments derived from a serial passage of three
consecutive transplantations of A549 cells were implanted subcutaneously
in nude mice. Xenograft mice bearing a tumor of 100 mm³ in volume were
treated with 12 mg/kg by bolus intravenous injection once daily. The
influence of P12 and P13 on the growth of A549 tumors was examined

and compared to effects exerted by a control ASO (HSV) or PBS alone. The administration of both ASOs P12 and P13 at a dose of 12 mg/kg over a period of 24 days revealed a significant effect on the growth of A549 tumors in mice (Fig. 10a). PLK1 ASOs administered systematically inhibited
5 the growth of A549 tumor xenografts in nude mice by 70% (P12) and 86% (P13), respectively. In contrast, no influence was observed when the tumor-bearing animals were treated with a control ASO (HSV) or PBS alone. The efficacy of ASO treatment on the inhibition of PLK1 expression in tumor cells was confirmed by an immunohistochemical analysis: While
10 administration of control ASOs (12 mg HSV/kg) for a period of 24 days had no significant effect on the frequency of PLK1-positive cells (76%), marked reduction of PLK1-positive cells (10%) was observed in P12- and P13-treated animals (Fig. 10b). Staining with the conventional marker for cellular proliferation MIB-I revealed a reduction from 61% to 8% of
15 immunostained proliferating cells in tumors.

Different experimental approaches had the goal to define the function of PLK1 in mammalian cells by altering the level of PLK1 expression. First, the inhibition of PLK1 function through antibody microinjection blocked
20 centrosome maturation in both nonimmortalized human Hs68 fibroblasts and HeLa cervical carcinoma cells (Lane et al., 1996). While PLK1-injected HeLa cells revealed severe mitotic defects such as immature centrosomes, nonimmortalized human Hs68 fibroblasts arrested in G₂ suggesting a centrosome-maturation checkpoint sensitive to alterations in PLK1
25 function. Second, results from transient expression of dominant negative PLK1 differed from previous antibody microinjection experiments in that most of the mitotic HeLa cells were bipolar and cytokinesis seemed to be disrupted (Mundt et al., 1997). Third, adenovirus delivery of a don-finant-negative PLK1 induced apoptosis in different tumor cell lines
30 (Cogswell et al., 2000). In contrast, normal human mammary epithelial cells arrested in mitosis, but seemed to escape the loss of centrosome maturation and mitotic defects observed in different cancer cells. Thus, the

use of different methods to abrogate the activity of PLK1 revealed its essential role for mitotic progression in mammalian cells. Interestingly, recent data extend previous observations by further showing that the majority of tested tumor lines are more sensitive to the inhibition of PLK1 function than normal epithelial cells (Cogswell et al., 2000). These results suggest that certain tumors show a selective apoptotic response versus normal epithelial counterparts. Still, the methods used in different studies are not suited for the systemic treatment of tumor-bearing animals or cancer patients. Antisense-based methods, which have been proven to inhibit the expression of certain critical cancer genes specifically, have already entered different clinical studies (Agrawal, 1996; Crooke et al., 1994; Zhang et al., 1995). The antisense drug Vitravene has now been approved for the treatment of patients with cytomegalovirus- induced retinitis (Crooke, 1998). Thus, we pursued the goal to test PLK1 as a possible target for cancer therapeutic intervention using an antisense oligonucleotide-based approach by reducing the endogenous level of PLK1 specifically in vitro and in vivo.

In contrast to many chemotherapeutical agents for the treatment of cancer cells, ASOs have the intriguing advantage to act specifically on the gene of interest. To test the specificity of our approach in detail, we analyzed the potential of different PLK1 ASOs to reduce the intracellular level of PLK1 expression and to act in an antiproliferative manner. As expected only very few ASOs were able to reduce the intracellular mRNA level, because due to the three-dimensional structure of mRNA only certain sequence domains are accessible for the heteroduplex formation with ASOs. Two ASOs P12 and P13 targeted against the 3'-untranslated region of human PLK1 mRNA were shown to be potent inhibitors of PLK1 mRNA and protein expression in cell culture. These ASOs displayed IC50 values of 50 nM. Interestingly, our experiments revealed an antisense response, which seemed to reflect the level of endogenous transcripts in the cancer cells examined: For example, the A549 cells with the highest frequency of expression showed

only a moderate response. Still, we can not rule out a homeostatic regulation, which can answer a decrease in mRNA concentration with a metabolic compensation. Administration of P12 and P13 to A549-tumor-bearing mice resulted in reduced PLK1 expression and in potent antiproliferative effects.

ASOs represent a potentially powerful method of selectively inhibiting gene expression. However, the study of antisense compounds has been hampered by some parameters resulting in variable experimental results. ASOs and in particular phosphorothioate versions used in many investigations are highly charged macromolecules that can induce toxic effects or might alter cellular functions via association to endogenous proteins which activate non-antisense mechanisms (Kuss et al., 1999). Biological activity attributed to such ASOs may not be the result of the specific inhibition of a target gene. Thus, demonstration of specificity is a key aspect for concluding true antisense action underlying the biological impact of a specific ASO. For this reason we paid considerable attention to the specificity of inhibition of PLK1 gene expression. Several experiments provided evidence for a specific mode of action by ASOs P12 and P13: (i) During an examination of 26 phosphorothioate ASOs targeted to different sites within the PLK1 mRNA, only a few candidates were potent inhibitors of PLK1 expression. (ii) Control ASOs (HSV-derived, nonsense) had no significant effect on PLK1 mRNA and protein expression in cell culture and in Xenograft experiments. (iii) Our data revealed that the suppression of PLK1 by ASOs was dose-dependent. In addition, we could demonstrate that the expression of other cellular genes like actin and p38 was not affected by P12 or P13 treatment.

The number of antisense cancer drugs that have entered clinical trials is increasing. At least four of these compounds are currently in phase 11 trials including those targeting protein kinase Galpha, bcl-2, c-raf and H-ras (Cunningham et al., 2000; Nemunaitis et al., 1999; O'Dwyer et al., 1999;

Waters et al., 2000; Yuen et al., 1999). Various studies suggest that the inhibition of early steps in signaltransduction causes the simultaneous activation of alternative, parallel signaling pathways resulting also in cellular proliferation. Thus, ASOs targeted against c-raf kinase led only to a transient inhibition of cellular growth (Monia et al., 1996). This effect might be due to the turnover of applied ASOs or due to the location of the raf protein in the signaling cascade. In contrast to early steps in signaltransduction represented by c-raf, ras, PKC, which are targets of antisense approaches, PLK1 is the first example of a kinase triggering terminal steps in the signaling cascade. Multiple observations provided evidence for a central role of PLK1 in the mitotic progression in mammalian cells which can not be by-passed on alternative routes of signal transduction (Cogswell et al., 2000; Lane et al., 1996; Mundt et al., 1997). Moreover, inhibition of PLK1-function was shown to induce tumor-selective apoptosis compared to normal epithelial cells. Considering these observations our experimental data suggest PLK1 as new target for cancer treatment. In addition to phosphorothioate ASOs, modified derivatives with altered sugar moiety or backbone which have improved pharmacokinetical and toxicological properties or low molecular weight inhibitors targeted against human PLK1 need to be developed for future therapeutic endeavors.

Oligonucleotides and antibodies

Phosphorothioate oligonucleotides (ASOs) were synthesized and purified by MWG Biotech (Ebersberg). Monoclonal PLK1-antibodies were obtained from Transduction Laboratories (Heidelberg) for Western blots and from Zytomed (San Francisco, USA) for kinase assays. Antibodies for actin were purchased from Sigma (Deisenhofen) and for p38 from Santa Cruz.

RNA preparation

For isolation of total RNA an RNA-easy-kit was used according to the manufacturer's protocol (Qiagen, Hilden). At least 5×10^5 cells were used
5 for preparation of total RNA.

Northern blot hybridization

Radiolabeling of the antisense strands of PLK1, β -actin and G3PDH was
10 performed using 250 μ Ci of [α - P^{32}]dCTP (6000 Ci/mmol; 1Ci = 37 GBq) for each reaction, 50 μ M of each other dNTP and 10 pmol (each) of primer PLK1-17-low (5'-tgatgttggcaccctttcagc-3'), actin-2-low (5'-catgaggtagtcagtcaggtc-3') and G3PDH-2 (5'-tccctgcctctactggcgct-3') as described previously (Hock et al., 1998). Northern blotting and
15 hybridizations were carried out as described (Wolf et al., 1997).

Cell Culture

DMEM and DMEM/F-12 1:1 mixture were obtained from Sigma, Ham's F12
20 and FCS from PAA. PBS, OPTIMEM, glutamine, penicillin /streptomycin and trypsin were purchased from Invitrogen. Tumor cell lines MDA-MB-435 and A549 were obtained from CLS (Heidelberg), HeLa S3 from DSMZ (Braunschweig) and cultured according to their instructions with slight modifications.

25

Western Blot Analysis

48 hrs after ASO treatment cells were lysed for subsequent Western blotting (Hock et al., 1998). Membranes were kept for 1 hr with
30 monoclonal PLK1 antibodies (1:250) and monoclonal actin antibodies (1:200.000) followed by incubation with goat anti-mouse antibodies (1:2.000) for 30 min. Western blots were performed as described (Bohme

et al., 1996).

Kinase Assays

5 Cells were lysed 24 hrs after ASO treatment for determination of PLK1
kinase activity. Endogenous PLK1 was immunoprecipitated using
monoclonal PLK1 antibodies (Zytomed) and then incubated with 0.5-1 μ g
of substrate and 2 MCi of [γ^{32} -P]ATP for 30 min at 37°C in kinase buffer
10 (20 mM HEPES pH 7.4, 150 mM KCl, 10 mM MgCl₂, 1 mM EGTA, 0.5 mM
DTT, 5 mM NaF, 0.1 mM Na₃VO₄). Kinase reaction was stopped and
fractionated on a 12% SDS-polyacrylamide gel (BioRad, München).

Indirect Immunofluorescence

15 Cells were stained as described before (Holtrich et al., 2000). Antibodies
were used as follows: monoclonal α -tubulin (Cedarlane, Canada) 1: 100,
polyclonal rabbit PLK1 (Yuan et al., 1997) 1: 100 or monoclonal γ -tubulin
(Sigma) 1: 100.

20 In vitro application of ASOs and determination of cell proliferation.

In vitro-inhibitory activities of ASOs on cell proliferation were tested by
transfection with different ASOs. Transfections were performed using the
DOTAP method (Roche, Mannheim). In brief, 1 day before transfection cells
25 were split to a density of $5 \times 10^5 / 25 \text{ cm}^2$ -culture flask corresponding to a
density of 40-50% at the time of transfection. Cells were treated with
ASOs at a concentration of 250 nM. After 3,5 hrs incubation with ASOs in
OPTIMEM with DOTAP at 37°C, transfection mix was replaced by normal
culture media. Cell numbers were determined at the appropriate time after
30 ASO treatment by direct counting using a hemacytometer. Cell viability
was assessed by trypan blue staining. Each experiment was performed at
least in triplicate and the standard deviation for each group was

determined.

Tumor xenograft studies

5 Human cancer xenograft models were established with at least 3 independent groups of 5 athymic nude mice (nu/nu) NMRI 8-10 weeks old (Harlan Winkelmann). For this purpose A549 cells were harvested, washed with PBS, resuspended in normal culture media and then 2×10^6 cells were injected subcutaneously into the animals flank regions. Arising tumors were
10 serially passaged by a minimum of three consecutive transplantations before the start of treatment. Then tumor fragments were implanted subcutaneously in both flanks of the nude mice and ASO application was started 25 days after transplantation when the tumor reached a volume of 100 mm^3 . ASO treatment (formulated in PBS) was carried out daily by
15 bolus injection ($100 \mu\text{l}$) into the animals tail vein at a dose of 12 mg/kg body mass for 24 days. Tumor diameters were determined using a caliper. Volumes were calculated according to the formula $V = 7t/6 \times \text{largest diameter} \times \text{smallest diameter}$. Standard deviations were calculated. Experiments were carried out in triplicate. After sacrificing the animals
20 tumors were excised for immunohistochemistry (Yuan et al., 1997).

Immunohistochemistry

Tumor sections of Xenograft tumors were prepared as described (Yuan et
25 al., 1997). Slides were incubated with monoclonal MIB-I antibodies (1:10; Dianova, Hamburg) and a polyclonal PLK1 antibody (1:200; Transduction Laboratories, Heidelberg). As detection system for PLK1 the EnVision+ System (Dako, Hamburg) was used according to the manufacturer's protocol.

Quantitative analysis

For semiquantitative analysis of Northern and Western blot signal intensities the autoradiographs were scanned using a Kodak gel documentation system (ID 3.5). After integration of signal intensities
5 expression of PLK1 and actin were correlated for quantitative comparison. The expression values were given in percentage of control. For quantitative evaluation of immunohistochemistry in A549 tumors, 10 high power magnification fields (400x) per slide were analyzed. Immunoreactive
10 staining of PLK1 and MIB-I is given in percent positive tumor cells.

Figure legends

Fig. 6. Reduction of PLK1 mRNA in vitro. (a) Relative positioning of the
5 predicted hybridization sites of the 26 tested PLK1 ASOs. Sequences of all
ASOs are available upon request. Arrows indicate positions of the potent
ASOs P12 and P13. (b) Northern blot analysis of PLK1 mRNA in
MDA-MB-435 cells 24 hrs after ASO treatment. HSV-ASO served as
negative control. To ascertain uniformity of loading the membrane was
10 reprobed with human B-actin and G3PDH. Percent inhibition was calculated
by comparison with standardized PLK1 mRNA levels of cells grown in the
absence of ASOs. PLK1 mRNA expression in HeLa S3 cells (c) and A549
cells (d) 24 hrs after ASO application. Membranes were reprobed with
human 13-actin.

15 Fig. 7. Inhibition of PLK1 protein expression and kinase activity by
PLK1-specific, ASOs in cancer cells. Control cells were incubated with
OPTIMEM. HSV-ASO served as negative control. Percent inhibition was
calculated by comparison with PLK1 protein levels in cells grown in the
20 absence of ASOs. (a) Western blot analysis of PLK1 protein in
MDA-MB-435 cells 48 hrs after ASO treatment. Indicated are the signals
for PLK1 and actin in both autoradiographs. Actin served as control for
equal loading (upper panel). Quantification of PLK1 protein levels after
normalization to actin was performed using a Kodak gel documentation
25 system (ID 3.5). Mean values of at least 3 independent experiments with
standard deviation are shown (lower panel). Quantification of PLK1 protein
in HeLa S3 cells (b) and A549 cells (e) after normalization to actin. PLK1
protein correlated to the concentration of ASO P12 in HeLa S3 cells (d) and
in MDA-MB-435 cells (e). PLK1 immunoprecipitated from ASO treated cells
30 phosphorylated casein to a lower extent compared to PLK1 from control
cells (upper panel). Coomassie staining served as control for equal loading
of substrate (middle panel) Equal amounts of PLK1 were subjected to the

enzymatic assay (lower panel) (f).

Fig. 8. Antiproliferative effects of PLK1 antisense inhibition in cancer cells. Control cells were incubated only with OPTIMEM. HSV-ASO served as a control. Percent inhibition was calculated by comparison to the number of cells treated with DOTAP grown in the absence of ASOs. (a) Growth inhibition of MDA-MB-435 cells treated with ASOs at a concentration of 250 nM over a period of 2 days. Growth inhibition of HeLa S3 (b) and A549 cells (c) over a period of 3 days. Dose-dependent reduction of MDA-MB-435 cells (d) and HeLa S3 cells (e) after ASO treatment. Mean values of at least 3 independent experiments with standard deviation are shown.

Fig. 9. ASO treatment induced abnormal centrosome distribution in A549 cells. Cells were transfected with PLK1 ASOs and immunostained 48 hrs post transfection for γ -tubulin. Interphase cells displayed abnormal localization of multiple centrosomes. Laserscan images (a and b) represent characteristic interphase figures.

Fig. 10. Effects of PLK1 ASOs on the growth of A549 tumors in nude mice. (a) Growth inhibition of two PLK1 ASOs (P12, P13) in contrast to control ASO (HSV)-treated and to untreated control mice. ASO administration was initiated 25 days after transplantation and continued for 24 days. Mean values of at least 3 independent experiments with standard deviation are shown (*: $p < 0.05$). (b) Immunohistochemical analysis of PLK1 and MIB-I in A549 tumor xenografts after treatment. A, PLK1 expression in ASO (I-ISV)-treated animals. PLK1 expression in P12-treated animals. C, MIBA expression in ASO (HSV)-treated animals. D, MIB-I expression in P12-treated animals.

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Ansprüche

1. Double stranded RNA (dsRNA) which is directed against PLK1.
2. Phosphorothioate antisense nucleotides (ASO) as described herein.
3. Use of antisense oligonucleotides and/or double stranded RNAs as claimed in claims 1 or 2 for the inhibition of proliferative diseases .
4. Pharmaceutical composition for the treatment of proliferative diseases, especially cancer, containing an effective amount of a dsRNA and/or an ASO according to claims 1 or 2.

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Abstract

5 The present invention concerns inhibition of the activity of PLK1, which
seems to be connected with cancer growth. Duplex RNAs and antisense
oligonucleotides have been found to be useful in such inhibition, therefor
they are claimed as ingredients fo pharmaceutical compositions for the
treatment of proliferative diseases, preferably cancer of various types.

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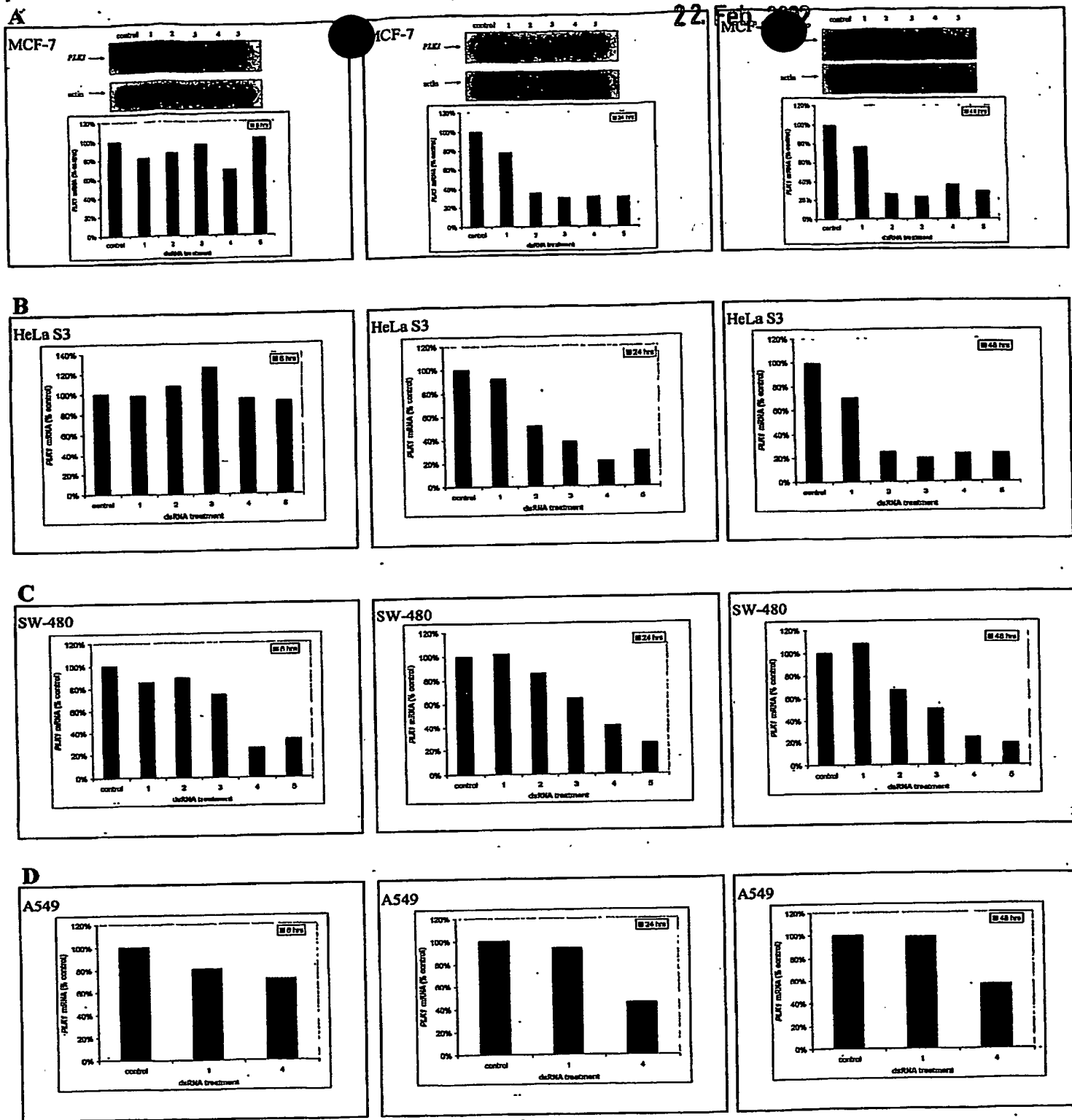


Fig. 1

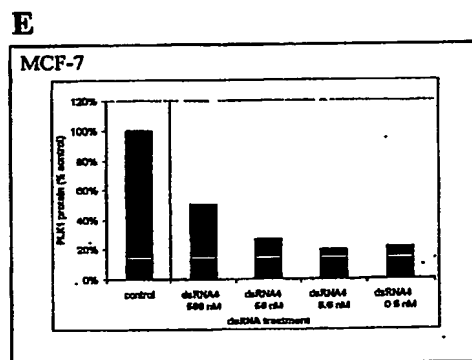
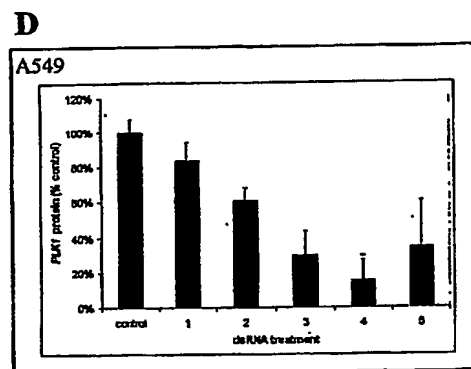
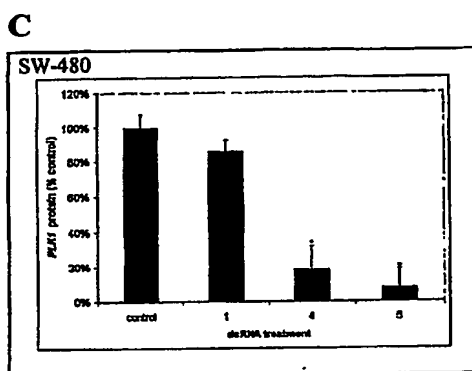
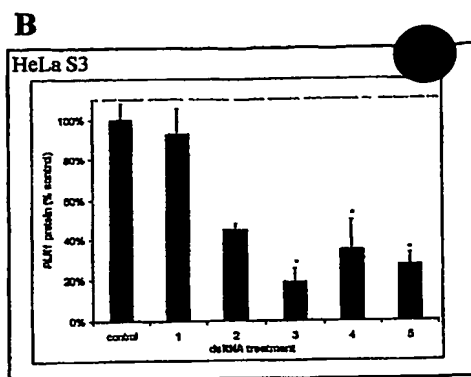
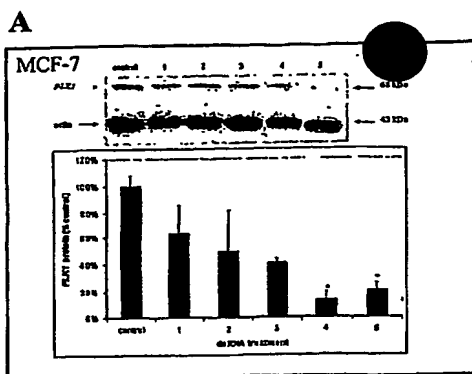


Fig. 2

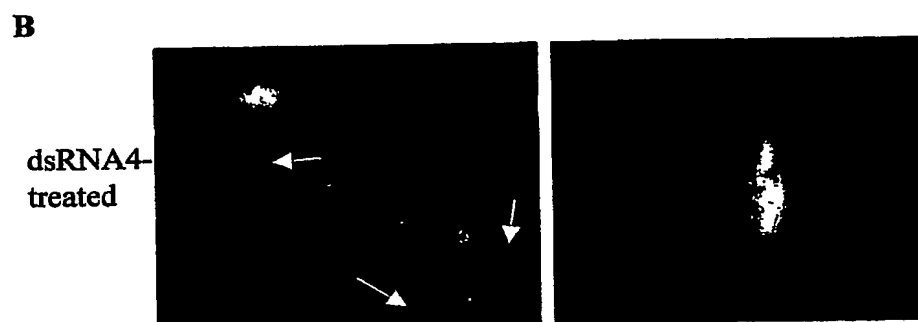
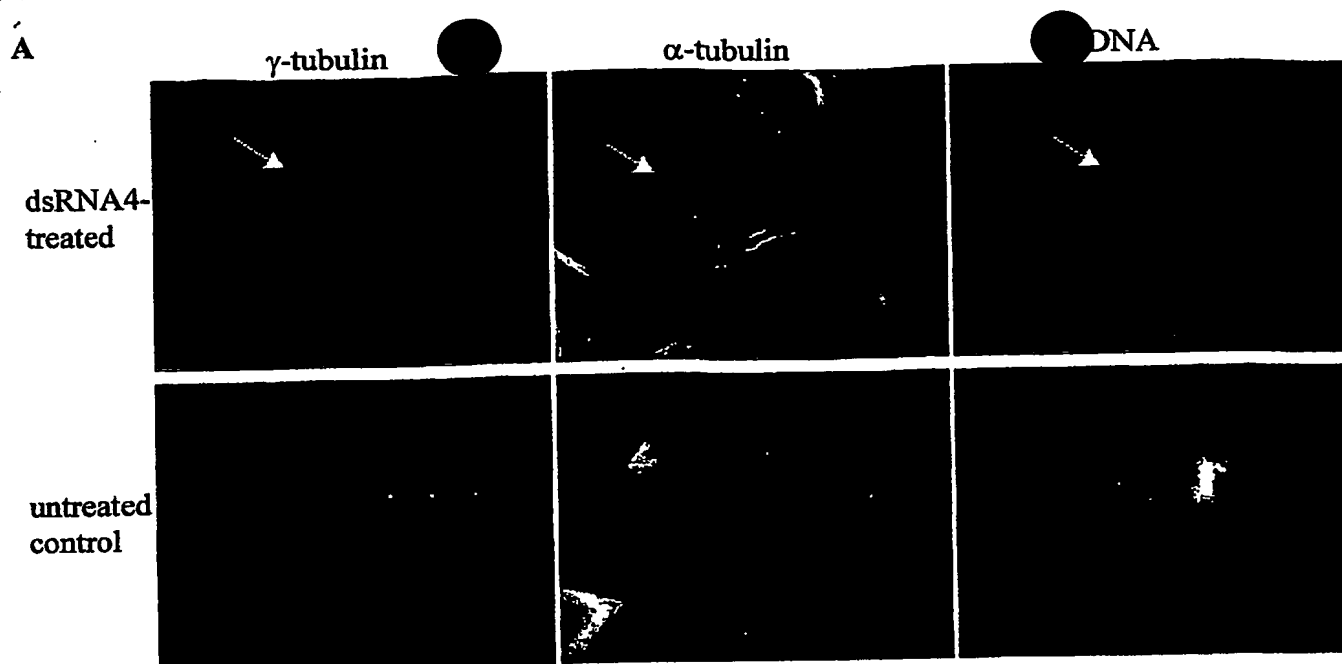
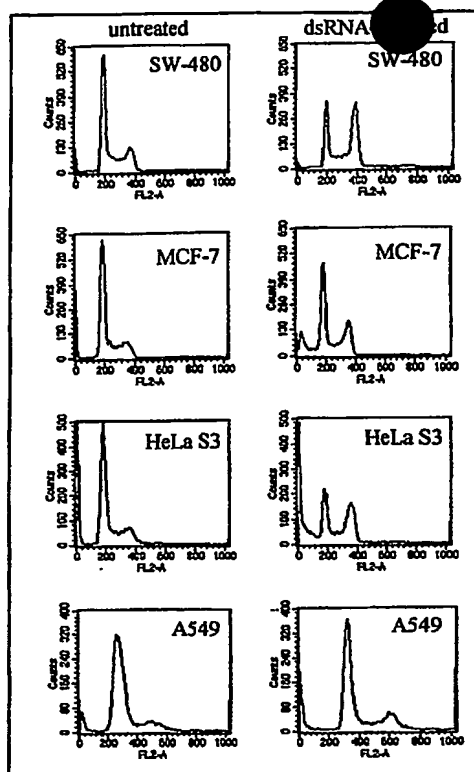


Fig. 3

A



B



Fig. 4

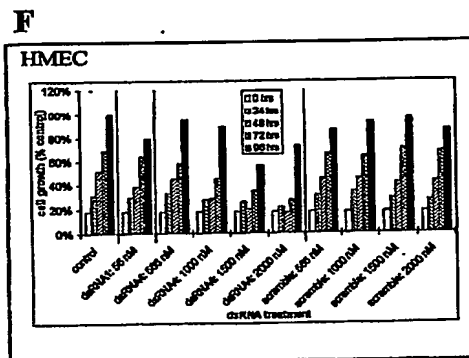
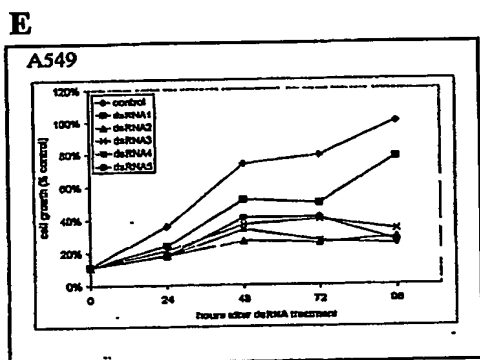
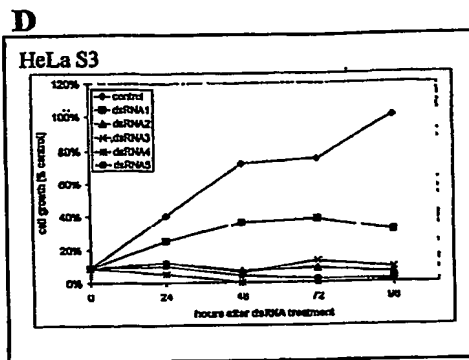
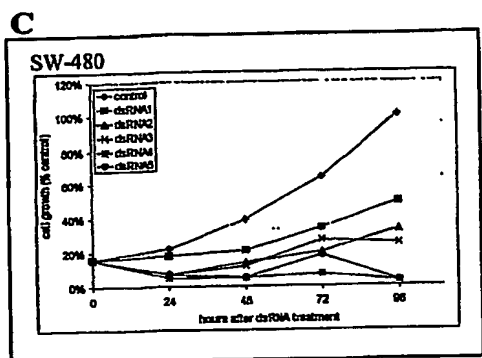
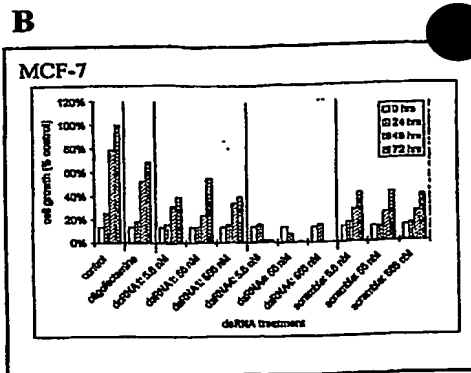
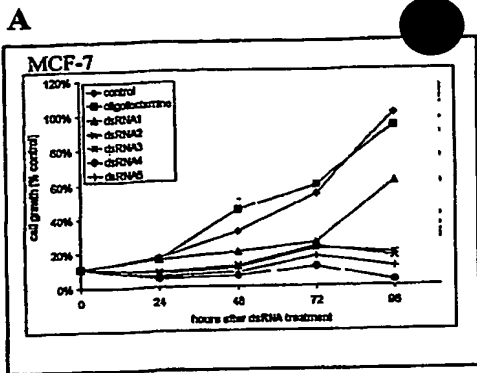
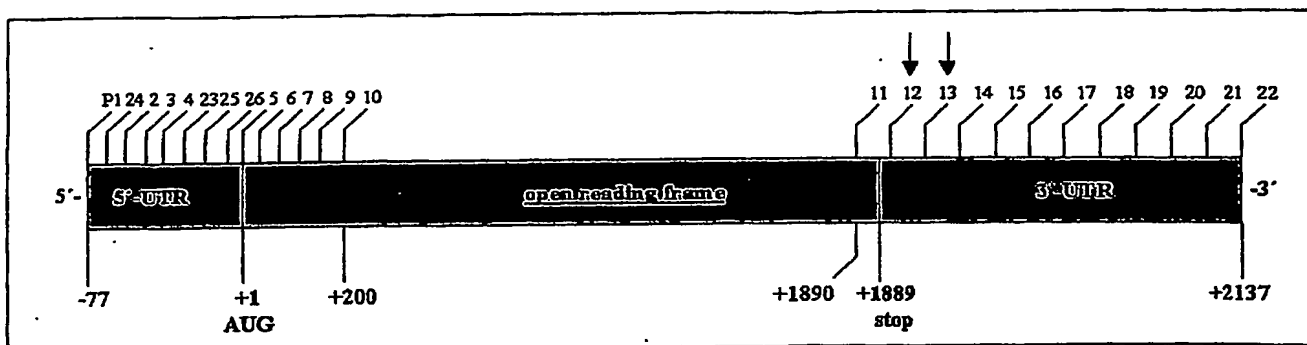
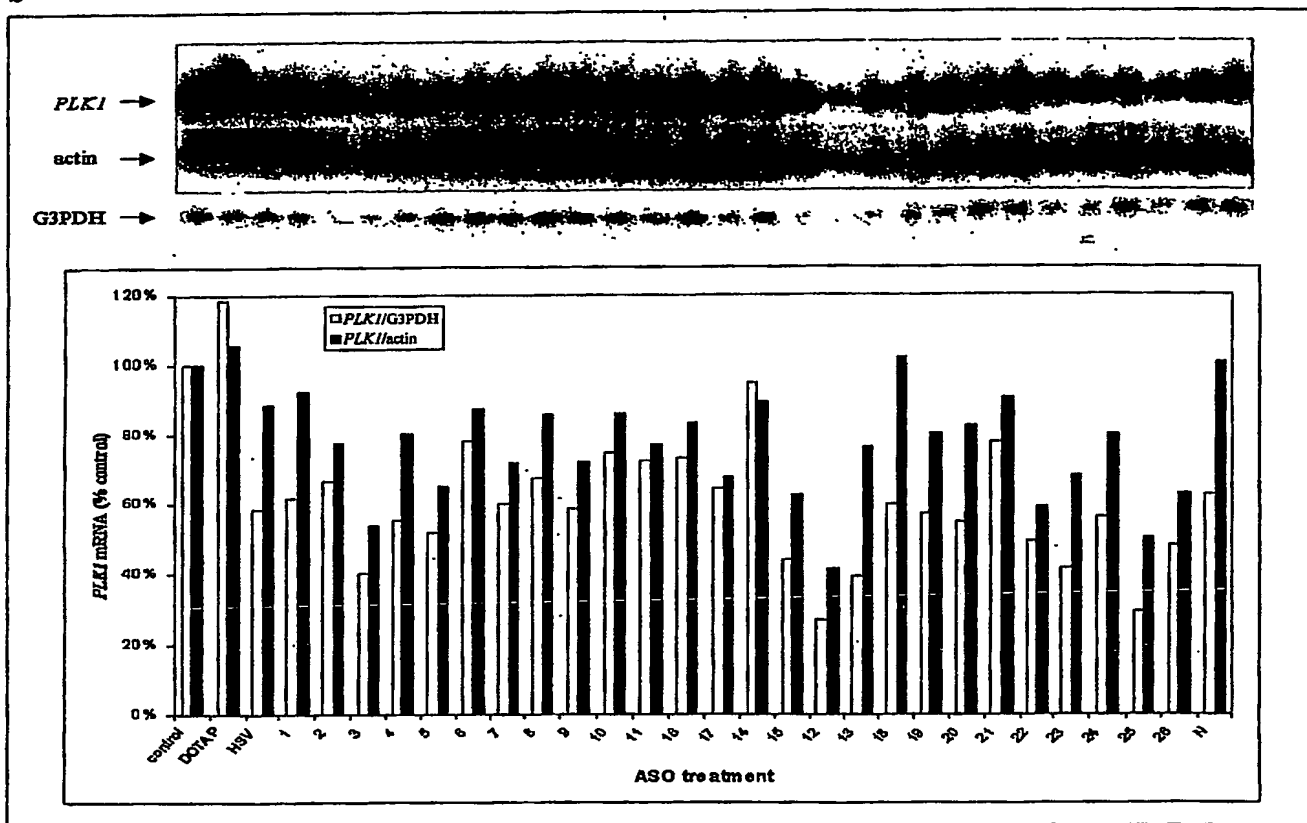


Fig. 5

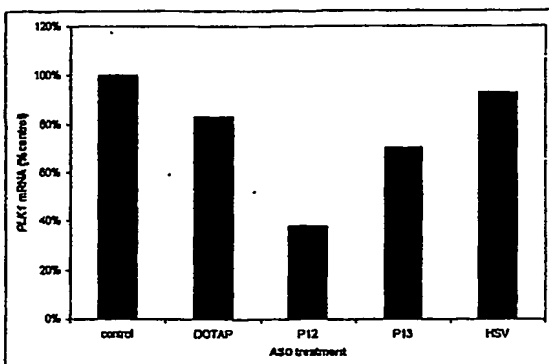
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b



c



d

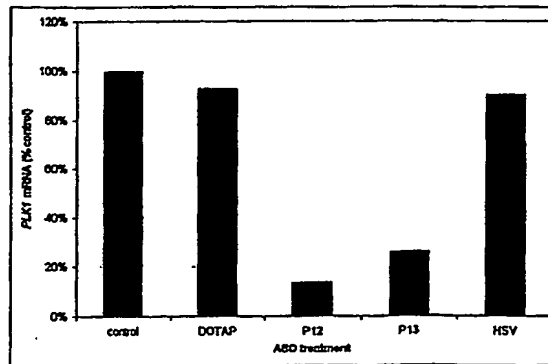


Fig. 6

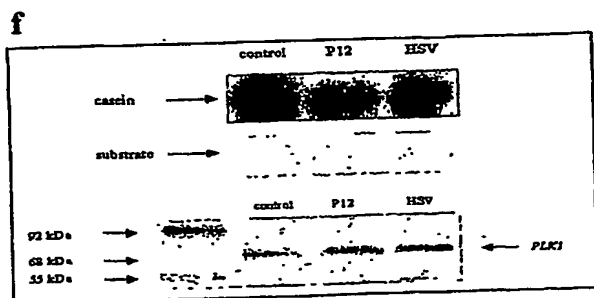
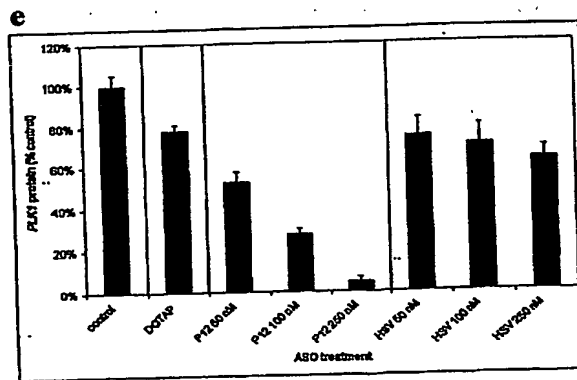
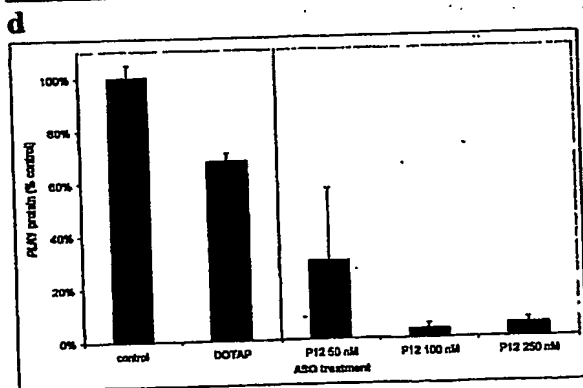
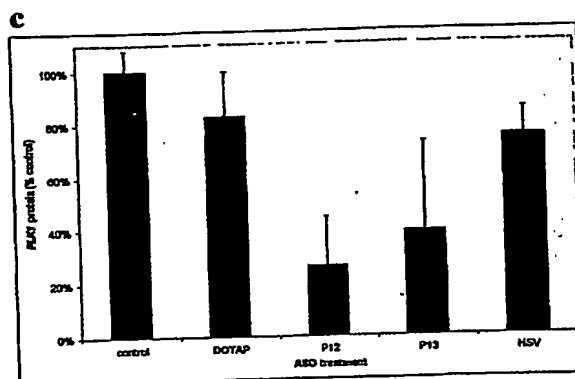
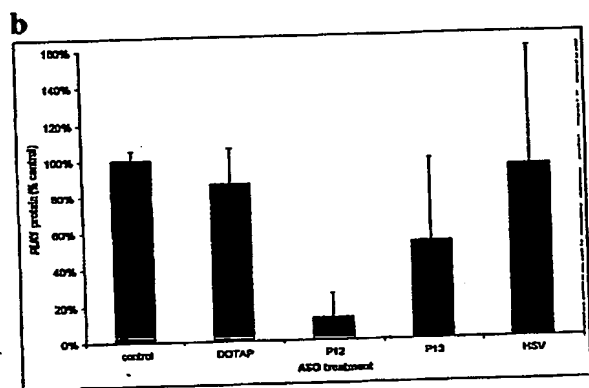
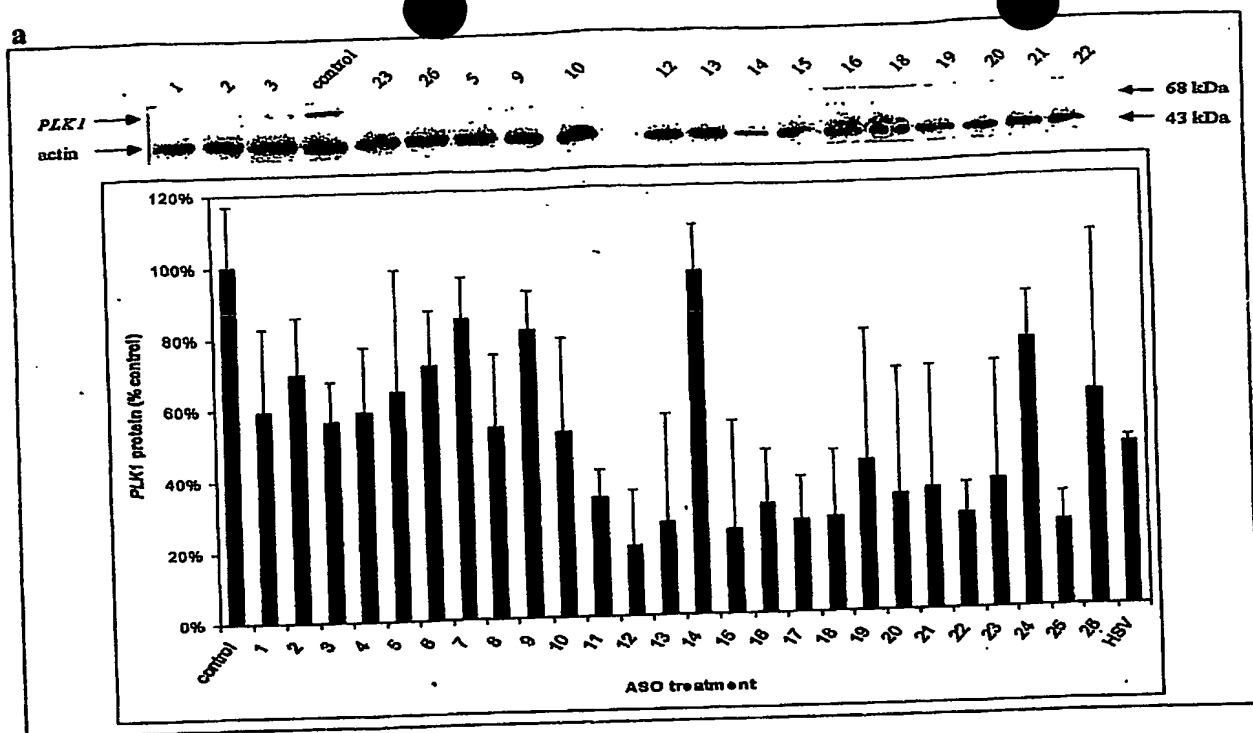
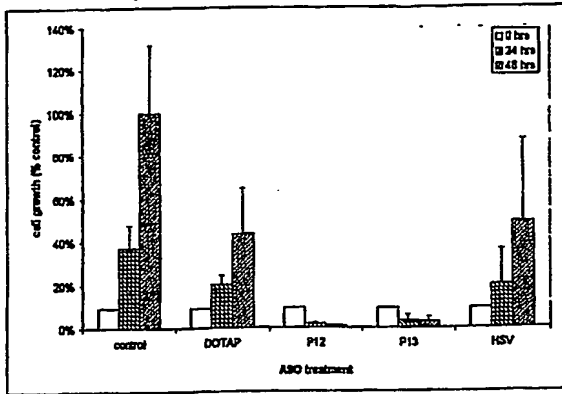
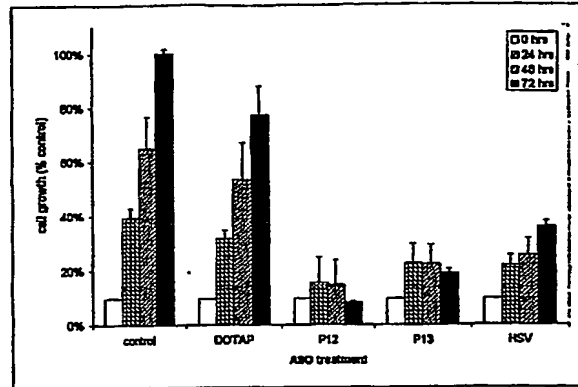


Fig. 7

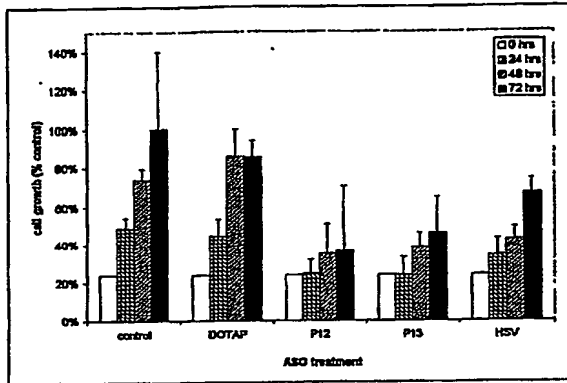
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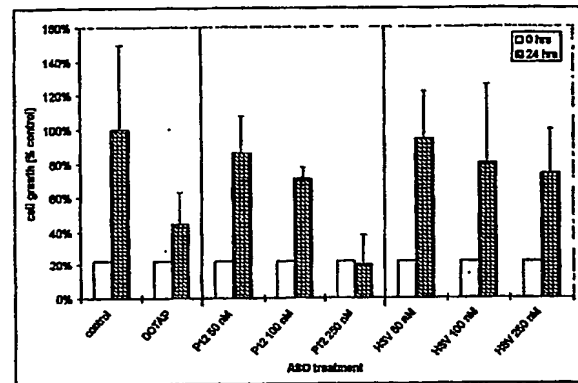
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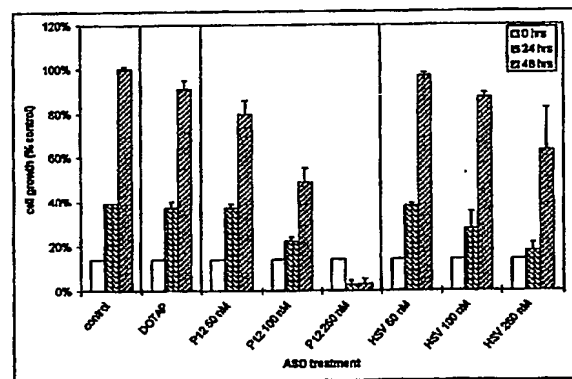


Fig. 8

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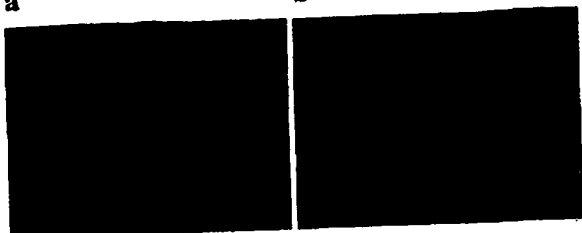


Fig. 9.

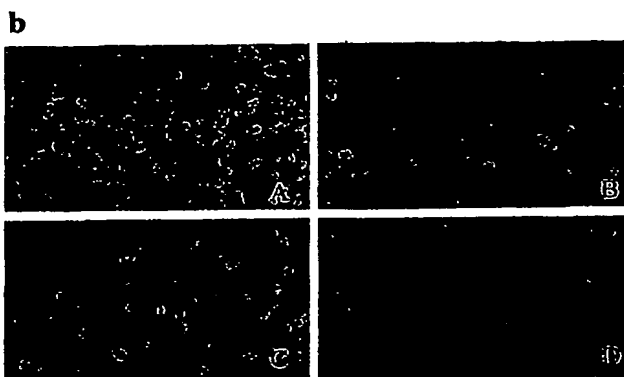
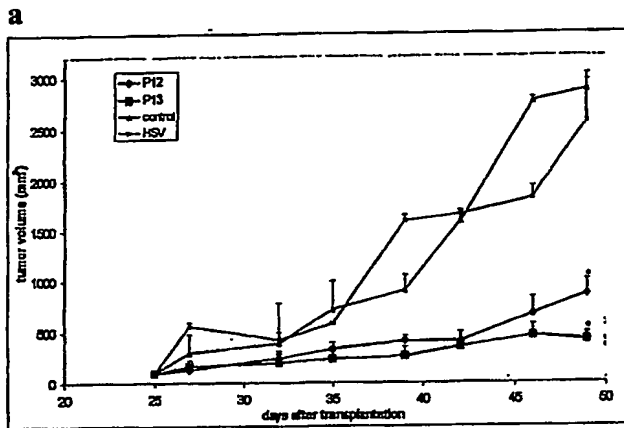


Fig. 10

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